The Challenge Posed by Endocrine-disrupting Chemicals

John Ashby,[1] Erik Houthoff,[2] Sandy J. Kennedy,[3] Jim Stevens,[4] Rémi Bars,[5] Friedrich W. Jekat,[6] Pamela Campbell,[7] Jim Van Miller,[8] Francis M. Carpanini,[9] Geoffrey L.P. Randall[10]

[1]Zeneca, Macclesfield, Cheshire, United Kingdom; [2]Akzo Nobel Chemicals, Amersfoort, The Netherlands; [3]Unilever, Bedford, UK; [4]Ciba-Geigy, Basel, Switzerland; [5]Rhône-Poulenc Agro, Sophia Antipolis, France; [6]Bayer Ag, Wuppertal, Germany; [7]Procter & Gamble, Strombeek-Bever, Belgium; [8]Union Carbide Corporation, Danbury, CT 06817-0001 USA; [9]European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels, Belgium; [10]Zeneca, Brixham, Devon, UK

Rapid regulatory developments in the area of environmental endocrine disruption present a series of potential problems that are identified and illustrated with examples taken from the recent literature. A list of priorities is provided, including the need for additional epidemiological and wildlife studies, the derivation of a coordinated testing strategy, agreement on the toxicities expected of endocrine disrupting agents, and acceptance that whole animal assays will be uniquely critical in this area of toxicology. The intrinsic difficulty of attempting to simultaneously study all aspects of endocrine disruption indicates the need to reduce the scope of the problem, which can be achieved by first studying toxicities mediated by sex hormone receptors. *Key words:* androgens, estrogens, hazard assessment, hormonal disruption, human toxicity, sperm quality, *Environ Health Perspect* 105:164-169 (1997)

It has been proposed that humans and wildlife have suffered adverse effects on reproductive health as a result of environmental exposure to chemicals that interact with the endocrine system (*1–4*). Mindful that a hypothesis is an idea that has not been sufficiently tested (*5*), many independent efforts have been undertaken to evaluate the scope and legitimacy of the problem. As one of these undertakings, the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) convened the present study group with the aims of discerning the degree to which synthetic chemicals are implicated in this issue and advising toxicologists and ecotoxicologists on appropriate testing methods and hazard identification strategies. After compiling a list of currently available test methods (*6*), we turned our attention to the identification of practical tests for endocrine disruption. To do that, we assumed that a range of endocrine disruption hazards are posed to wildlife and humans by environmental chemicals. We recognize the importance to any future regulatory initiatives in this area of demonstrating dose–response relationships, establishing the relevance of experimental models to humans and wildlife, and assessing exposures. However, these tasks were considered to be beyond our initial purpose.

In support of attempts to develop an appropriate hazard identification strategy for endocrine-disrupting chemicals, several regulatory initiatives have been launched, the most specific being a mandate by Congress that the EPA should have a regulatory framework on endocrine disruption in place by 1998 (*7*). Such a condensed time frame carries with it the potential for the premature endorsement of unvalidated assays and unrefined testing strategies. This article outlines some of these potential problems with a view to their clear recognition and circumvention.

The Potential Problems

Kavlock et al. (*8*) recently conducted a definitive review of data that are cited to support links between a range of human health effects and exposure to endocrine-disrupting chemicals. The key conclusions of that analysis are presented in Table 1. These authors concluded that there are no clear relationships between endocrine effects in humans and exposures to xenobiotics. These conclusions are further dissected in Table 2 according to the criteria recommended by Hill (*9*) for distinguishing between epidemiological association and causation, as recommended by Kavlock et al. (*8*). For comparative purposes, the data supporting causation for the 56 established human carcinogens are also presented. That analysis reveals only tentative associations between human exposure to chemicals and the observation of any of the endocrine toxicities listed in Table 1; with the exception of the clinical use of diethylstilbestrol (DES), there are presently no proven causations in humans. There are also no data to support the assumption that synthetic chemicals, as opposed to naturally occurring chemicals (and in the case of humans, dietary constituents, lifestyle, etc.) are the most important etiologic contributors to the projected problem (*1*). A similar assumption, now considered to be incorrect (*10*), was made in the early stages of the study of environmental carcinogenesis. This all emphasizes that any moves to regulate chemicals showing endocrine-disrupting properties should be cautionary and based on confirmed evidence, all of which currently derives from either wildlife or experimental studies. Specifically, the justification for any future regulatory actions should not be based on the presumption that such moves will automatically alleviate the human health effects discussed by Kavlock et al. (*8*).

In contrast to the situation in humans, several etiological links between exposure to synthetic endocrine-disrupting chemicals and adverse effects on wildlife have been established, mainly in contaminated environments (*1–4*). Nonetheless, the data supporting some of the suspected environmental links are as fragile as those noted by Kavlock et al. (*8*) for human effects. An example of this is provided by the predominant role played by the natural hormones estrone and 17β-estradiol, as opposed to synthetic xenobiotics, in the partial feminization of fish exposed to effluent of some municipal sewage treatment plants in the United Kingdom (*11–16*).

Given that there is significant conservation among animal species in the mechanisms that control sexual reproduction and development, it is suggested that it will be possible to adopt, at least initially, a common strategy for the identification of wildlife and human endocrine disruptors. For example,

Address correspondence to F.M. Carpanini, European Centre for Ecotoxicology and Toxicology of Chemicals, Avenue E. Van Nieuwenhuyse 4, B.6, B-1160, Brussels, Belgium.

The members of the study group considering the issue of endocrine disruption are: G. Randall (Chair), Zeneca plc; F.M. Carpanini (Secretary General), ECETOC; J. Ashby, Zeneca plc; R. Bars, Rhone-Poulenc Agro; P. Campbell, Procter & Gamble; J. Cooke, DuPont (U.S.); C. De Rooij, Solvay; K. Eide Lunde, Norsk Hyrdro a.s.; P. Foster, Chemical Industry Institute of Toxicology; C. Hegele-Hartung, Schering; E. Houthoff, Akzo Nobel Chemicals; M. Hurtt, DuPont (U.S.); T. Hutchinson, Zeneca plc; J. Jackson, Monsanto Europe S.A.; F.W. Jekat, Bayer Ag; S. Kennedy, Unilever; R. Länge, Schering Ag; P. McCahon, Rhône-Poulenc (Secteur Agro); N. Moore, BP Chemicals Ltd.; A. Nikiforov, Exxon Biomedical Sciences, Inc.; D. Pallapies, BASF Ag; A. Poole, Dow Europe; A. Sarrif, Du Pont (Deutschland) GmbH; N. Scholz, Huls Ag; J. Stevens, Ciba-Geigy; L. Tattersfield, Shell Research and Technical Center; J. Tomenson, ICI Epidemiology Unit; R. van Egmond, Unilever; J. Van Miller, Union Carbide Corporation.

Received 13 December 1996; accepted 9 January 1997.

Table 1. Conclusions drawn by Kavlock et al. (*8*) in relation to the data supporting endocrine-disrupting chemicals producing the known or suspected human toxicities listed

Possible human endocrine toxicities	Conclusions drawn by Kavlock et. al (*8*)
Cancer	
Breast	• DDT/DDE/PCBs—conflicting data regarding their etiological involvement • EPA database on pesticides—no alerts to estrogenic mammary gland carcinogenesis • 450 NTP studies; 10% of studies induce mammary gland cancer, but chemicals unlikely to be estrogenic • Concomitant decline in uterine and male breast cancer observed
Testicular, ovarian	• No evidence that endocrine-disrupting chemicals are a risk factor
Reduced sperm quality	• The generality of the effect and its etiology are far from certain
Reproduction	• First priority: hypotheses generated from field observations must be tested
Neurological	• First priority: studies to define the biological effects most likely to occur must be conducted
Immunological	• Studies to determine if there has been an increase in cases of immune dysregulation should be conducted

Abbreviations: PCBs, polychlorinated biphenyls; EPA, U.S. Environmental Protection Agency; NTP, National Toxicology Program.

Table 2. The nine criteria suggested by Hill (*9*) for distinguishing associations and causations in epidemiological studies, as applied to the two areas shown [a]

Hill criteria for establishing causation	The 56 chemical carcinogens to humans	Chemical endocrine toxicities in humans
Strength (of evidence)	+	–[b]
Consistency (of evidence)	+	–
Specificity (of effect)	+	–
Temporality (of effect)	+	–
Dose response (of effect)	+	–
Plausibility (of effect)	+	+
Coherence (with existing knowledge)	+	+
Experiment (simulation in rodents)	+	?
Analogy (structure activity)	+	?

+, criteria for causation met; –, criteria for causation not met; ?, criteria partially met.
[a]Structure–activity relationships are poorly defined in endocrine toxicity. Many endocrine toxicities have been produced in rodents, but these do not necessarily simulate effects in humans as only diethylstilbestrol is available for study.
[b] Except diethylstilbestrol.

the fact that a chemical can induce the production of the female-specific protein vitellogenin in male fish raises the possibility that it may cause corresponding effects in other wildlife and in appropriately exposed humans. Likewise, the activity of an agent in a rodent uterotrophic assay contributes to assessment of its potential to cause endocrine toxicities in wildlife. Differences between the assessment of wildlife and human toxicities will most likely be encountered at the level of the nature, timing, duration, and magnitude of exposures, i.e., at the level of risk assessment. On some occasions, differences in the physiology or biochemistry of the reproductive process between species or differences in the accumulation of chemicals between the major environmental compartments may become important risk modifying factors. However, it is suggested that such differences (e.g., the possibly unique effects of chemicals on metamorphosis or molting) should be specifically studied to determine their importance, rather than being used to fragment the study of endocrine disruption at this early stage.

Definitions and Terminology

A surge of studies to define chemical endocrine disruptors, coupled with attempts to solve problems created by preliminary studies, is leading to a confusion of terms. For example, agents are already being labeled as estrogenic based on their activity in the MCF-7 assay (the E-SCREEN) (*17*) despite the fact that a range of non-estrogenic factors can stimulate these cells to divide (*18*), and the fact, common to all of biology, that not all activities observed *in vitro* are realized *in vivo*. Alternatively, the absence of agreed-upon definitions could lead to unrelated activities of a chemical being linked with the implication of a mechanistic association. For example, it is superficially attractive to assume that the activity of butylbenzyl phthalate (BBP) in the MCF-7 assay (*17*) is directly predictive of its reported ability to reduce testis weight in rats (*19*). However, BBP and its principal metabolites are inactive in immature and ovariectomized rat uterotrophic assays [(*20*); M.A. Martens, unpublished data], and its activity as a testicular toxin remains to be confirmed and explained. Similarly, the observation that continuous subcutaneous infusion of nonyl phenol is capable of stimulating cell division in female rat mammary tissue (*21*) was suggested to be unrelated to its uterotrophic activity in the rat following intraperitoneal injection (*22*), and the ability of the anti-estrogen raloxifene to counter bone density loss in ovariectomized rats (*23*) was recently shown not to involve the DNA-binding domain of the estrogen receptor with which this chemical is known to interact (*24*). These recent examples illustrate the complexity of the biological issues being approached, and they serve to warn against the precipitate adoption of simplistic definitions and testing strategies.

It is therefore suggested that there is a need for a set of toxicological definitions that will serve this new area. For example, there are two current definitions of an estrogen—a compound that binds to isolated estrogen receptors, and a compound that produces trophic effects on the female reproductive tract. However, what is required is a definition of the toxic effects expected of exposure of a whole organism to such a chemical, a definition that may differ between species and sexes. In the absence of such guidance it will be easy to drift into hypothesis-fulfilling conclusions. For example, an agent may show evidence of potential estrogenic activity by virtue of its activity in one of the many available *in vitro* assays, and then be administered to animals to confirm the expression of this toxic potential *in vivo*. However, if there are no agreed-upon expected toxicities, the chemical's ability to affect kidney weights or thyroid gland function, for example, may be taken as automatic confirmation of the original prediction. Such empirical associations may mislead, as illustrated by the three examples cited above.

The collective term endocrine disruptor is coming into general use, but it has yet to be defined. We suggest that an endocrine disruptor should be defined in reference to an intact endocrine system, i.e., as an agent that can induce adverse health effects in an intact organism, consequent to disruption of the organism's endocrine system. Other potentially relevant properties of the chemi-

cal, including any effects observed *in vitro*, can only contribute to its definition as a potential endocrine disruptor. Specifically, the activity of a chemical in any of the available *in vitro* assays does not define it as an endocrine disruptor, any more than does the demonstration of a chemical's ability to, say, inhibit the enzyme aromatase *in vitro*.

Rodent Toxicities of Concern and Reference Chemicals

A precondition for framing toxicological definitions is the existence of a list of sentinel rodent toxicities related to each type of endocrine disruption, together with a database of reference positive control chemicals for the toxicities named, as developed in other branches of toxicology (*25,26*). Despite their clear importance, neither of these lists has yet been finalized by the scientific community. In the case of estrogenicity, estradiol or DES are available as positive control agents, but even in these cases the estrogen-specific toxicities expected are not universally agreed upon.

In the absence of agreement about a range of reference endocrine disruptors, it will be difficult to assess the general sensitivity of existing or emerging tests. Equally, the current absence of agreement on chemicals that are inactive as endocrine disruptors makes it impossible to evaluate the specificity of emerging *in vitro* assays, or to discern the lower level of activity in any predictive assay that would be expected to lead to significant endocrine toxicities *in vivo*. This absence of *in vivo* toxicity data for a range of toxic and nontoxic reference chemicals must be remedied in order to have a sound research foundation upon which to build an effective regulatory strategy. Individual research groups or regulatory authorities may know of such reference chemicals, but unless these can be shared with the general scientific community, they effectively do not exist.

Structure–activity Relationships

The derivation of structure–activity relationships (SARs) in this area would aid the prioritization of chemicals for testing. However, McLachlan (*2*) and Katzenellenbogan (*27*) have pointed out the current difficulty of reconciling chemical structure with the wide range of chemical substances reported to have one or other of the several different endocrine-disrupting properties. It is anticipated that useful SARs will exist in situations where specific effects are studied within discrete chemical series, using standardized bioassays and clear criteria for activity. Waller et al. recently described one such approach (*28*). Nonetheless, it is important to acknowledge that SARs derived from *in vitro* studies may differ significantly from SARs derived from studies conducted *in vivo*. Furthermore, it is unlikely that any general SARs capable of encompassing all categories of endocrine disruptors will be developed.

The report that intraperitoneal injection of high dose levels of the solvents ethylene glycol and dimethyl formamide to rainbow trout resulted in increased levels of vitellogenin mRNA (*29*) was unexpected, given that these chemicals are structurally remote, on all counts, from the endogenous estrogen receptor agonist estradiol. The data in question could indicate one of three possible things, each of which is pertinent to the construction of SAR databases for endocrine disruption. First, if they are taken as evidence of the estrogenicity of these two chemicals, they illustrate the current absence of understanding of the critical chemical features required for endocrine-disrupting activity. A second possible explanation is that these chemicals activated the estrogen receptor by inducing a change in its conformation, as opposed to binding to the receptor. If this explanation were confirmed, it would open up a new area of chemical estrogenicity whose toxicological significance is presently unclear, and which would require the derivation of a separate SAR database. A third possible explanation is that changes to vitellogenin mRNA levels, observed without confirming commensurate changes in protein levels, may not provide a reliable indicator of the estrogenic activity of chemicals. Determination of which of these three explanations is correct will require further studies, and, prior to the conclusion of those studies, it would be inappropriate to enter either of these two solvents into any SAR database. This discussion therefore reverts to the critical need for agreed-upon definitions of endocrine activities as a precursor to the derivation of SARs and assays for those activities.

In Vitro Assays

The crucial problem is that there are no standard criteria for the selection, development, or grouping of assays into test batteries or tiers, or for assessing their sensitivity, specificity, and relationship to each other. Such questions need immediate attention, because chemicals are already showing different qualitative responses between similar *in vitro* assays, between *in vitro* and *in vivo* assays, and between different routes of administration *in vivo* (*30*). For example, several yeast assays having the human estrogen receptor stably integrated into their genome are in current use. These assays have subtly different reporter gene constructs and variable numbers of estrogen response elements, and it is currently unclear what effect, if any, these differences will have on experimental outcomes. Similarly, many laboratories are using one or more of a variety of transiently transfected receptor cell lines, and some of these may be difficult to transfer into routine regulatory use (compare the level of standardization achieved rapidly, and essentially, for the *Salmonella* mutation assay). Finally, some of the currently available *in vitro* assays, although potentially valuable, are complex and/or time consuming to conduct, as illustrated by the fish primary hepatocyte vitellogenin assay (*31*). The present proliferation of *in vitro* assays will inevitably continue apace with revelations of the complexity of normal sexual reproduction (*32*). Therefore, prior to the formal, regulatory adoption of any of these assays, it is vital that the differences between existing assays be elucidated and critically examined, robust versions of the preferred and validated assays be developed for routine use, and an agreed-upon framework in which these assays should be used be derived. Failure to meet these needs will lead to delays in effective implementation similar to those that accompanied the introduction of mutagenicity assays.

Among the *in vitro* assays so far described, with the obvious exception of the fish hepatocyte assay and the possible exception of the receptor-based yeast assays, none appear to be metabolically competent. The use of *in vitro* mutagenicity assays in the absence of liver enzymes, e.g., S9 mix, would lead to the nondetection of many mammalian mutagens and carcinogens, and a similar problem should be anticipated in this area. For example, Shelby et al. (*33*) reported that the *in vivo* xenoestrogen methoxychlor is unable to bind to isolated estrogen receptors or activate those receptors in a mammalian cell transactivation assay. This observation led to the independent study of the same sample of methoxychlor in a yeast human estrogen receptor transactivation assay, with the goal of confirming its inactivity and establishing the importance of auxiliary metabolism. In fact, it was found to give a potent direct-acting positive response in the yeast assay, presumably reflecting the ability of the yeast cells to demethylate the methoxy groups yielding the active estrogenic phenol derivative (*34*). This example confirms that the issue of metabolism *in vitro* has the potential to confound the validation of mammalian cell *in vitro* assays.

In addition to the general problem of metabolism and, again, based on experience gained with mutagenicity assay devel-

opment, it will be helpful if investigators can rapidly agree which assays are unreliable or nonspecific, and then share that conclusion openly. As an example, the polyclonal nature of MCF-7 cells (*17*) and the insensitivity of some of the clones to estradiol (*35*), coupled with the problem of the assay's low specificity, suggest that this assay will have limited value for general screening purposes, despite the fact that it can be performed adequately in some laboratories. There is a need for such a clear conclusion to be openly agreed upon in the scientific community because, in its absence, the assay will continue to be used to define potential endocrine disruptors. All new test systems should be scrutinized by the broader scientific community before they are accepted for general use.

It is proposed that the development of *in vitro* assays for potential endocrine disruptors should be led by the naming of significant toxicities that are consequent to disruption of the endocrine system of intact organisms, followed by attempts to model these effects *in vitro*. When appropriate, such assays should then be refined to produce robust test protocols suitable for general use. This is in contrast to the uncoordinated proliferation of superficially validated assays that act as a brake on progress and lead to the generation of potentially large amounts of uninterpretable data. The need for scientific caution in progressing this new area of toxicology is illustrated by the failure to confirm (*35,36*) the recent report by Arnold et al. (*37*) of synergism of estrogenic activity observed *in vitro* between a range of environmental chemicals.

In Vivo Assays

It is general practice in toxicology to screen for a potential toxic activity *in vitro* and to then confirm the expression of that activity *in vivo* before attributing a given toxic property to the test agent. For this and several other reasons outlined in this article, *in vivo* assays will assume a dominant position in screening strategies and risk assessment processes for endocrine disruption. Further, the trend common to other branches of toxicology of combining a range of end points in a single test protocol probably will apply equally to *in vivo* assays for endocrine disruption. However, an inevitable corollary to the use of multiple-end point assays is that one is forced to rank end points, often in the absence of guiding data, when qualitatively divergent responses are obtained among the several end points being monitored. This indicates the need for an established hierarchy of endpoint sensitivities for studying endocrine disruption in a given organism. In addition, the decision to conduct an assay *in vivo* carries with it a range of decisions regarding the choice of test species and strain, route of administration, and duration of dosing. The rodent uterotrophic assay illustrates why these generic problems should be discussed before, rather than after, the regulatory protocols for *in vivo* assays are fixed. The uterotrophic assay is often referred to as the gold standard of estrogenic activity *in vivo*. However, the data upon which this reputation is based were derived using a variety of protocols. The key variables were the use of rats or mice; the use of immature, hypophysectomized, or ovariectomized animals; the use of subcutaneous, intraperitoneal injection or oral administration of the test agent; and a dosing duration of between 3 and 6 days. Furthermore, some investigators recommend concomitant assessment of associated markers of estrogenic activity, such as vaginal opening, vaginal cornification, uterine epithelial cell height, or stromal proliferation (*38,39*).

To decide which of these many variables are important to the overall sensitivity of an assay will require assessment of a range of appropriate positive and negative endocrine disruptors. Similarly, it will be important to study the sensitivity and specificity of proposed estrogen action markers before they can replace existing markers. For example, lactoferrin mRNA levels in the immature mouse uterus can be increased several hundredfold when exposed to an estrogen (*40*), but before this can be developed into a replacement for the uterotrophic assay, it will be necessary to evaluate the specificity of this response, and to establish that the estrogen and growth factor response elements in the mouse uterus lactoferrin gene are representative of that in humans. The failure to broach such basic questions in genetic toxicity research has led to the development of a large number of competing *in vivo* techniques with no general agreement on which of them are complementary to other assays and which are redundant.

Multiple Mechanisms of Action

Agreement on a testing strategy to detect significant mammalian and wildlife estrogens would be relatively easy to achieve, and several such proposals have already been made (*38,39,41*). However, such a strategy would not be expected to predict endocrine toxicities associated with disturbances to normal steroid hormone synthesis or metabolism, thyroid gland function, or pituitary and hypothalamic feedback control mechanisms. Such effects will be difficult, if not impossible, to simulate *in vitro*, and this again indicates the need for a high level of reliance on acute or subacute whole organism assays. For example, although some of the *in vivo* effects of PCBs may be predicted by *in vitro* assay results, particularly those effects mediated by direct receptor interactions, this will not always be true. As an example, it is unlikely that any cell-based assay could anticipate, at least for the correct reason, the ability of certain PCBs to increase the weight of rat testes (*42*). This is because the effect is dependent upon PCB-induced hypothyroidism preventing the cessation of Sertoli cell division on about day 16 postpartum, an effect that is probably independent of the weak uterotrophic activity seen for PCBs in the rat (*43*). Likewise, the testicular effects reported for BBP (*13*) are unlikely to be associated mechanistically with its mitogenicity to MCF-7 cells, just as the endocrine toxicities of *p,p'*-DDE are most probably mediated by its antiandrogenic properties, rather than by its initially defined estrogenic properties (*44*).

Differentiation of Toxicities and Effects

The present uncertainty regarding the *in vivo* toxicities to expect of a chemical that has shown activity *in vitro* could lead to the measurement of a wide range of parameters in follow-up *in vivo* studies. Such toxicological fishing exercises might sometimes be justified, but to be useful they will require the separate recognition of significant toxicities and transient adaptive effects. For example, a small chemically induced change in the levels of sex hormones in a rodent may be devoid of toxic significance. A different example is provided by the measurement of the anogenital (AG) distance in neonatal rats whose mothers have been exposed to a potential endocrine-disrupting agent. This end point is a potentially valuable marker of endocrine disruption, but it has been little studied to date, and few control data have been published. Therefore, it is legitimate to interpret with caution alterations in this parameter in cases where the effect resolves by weaning and the pups show normal sexual development and function. Such effects may be of value to explain the observation of a recognized endocrine toxicity, but they may be of little value when the effects themselves constitute the only evidence for endocrine disruption. This is in contrast to situations where an irreversible change in AG distance is subsequently accompanied by other effects, such as a change in the time of vaginal opening or preputial separation, or reduced fertility of the adult rodents. This may change with the acquisi-

tion of a larger database for AG distance and other relatively new markers of endocrine disruption, but in the meantime it is dangerous to interpret such effects in isolation. A related example would be a transient induction of the mRNA in male trout that was not shown to be accompanied by the synthesis of vitellogenin protein. Distinguishing toxic responses from transient effects will be particularly important in these early days of the study of endocrine disruption, because it will enhance the rapid recognition of endocrine toxicities of immediate potential relevance to humans or wildlife.

An Integrated Approach

If the current concerns turn out to be justified, the problem posed will not be confined to a few countries. Therefore, it is important that the many initiatives being undertaken by individual governments and chemical industries to assess this issue be prioritized and developed with some level of international coordination.

Steps required for the effective regulation of endocrine-disrupting chemicals:

- International liaison
  - Coordinate studies to verify the suspected chemically mediated endocrine toxicities in wildlife and humans.
  - Share research plans.
- Prioritization of areas of study
  - Specification of key endocrine disruption toxicities and mechanisms and the compilation of a database of appropriate endocrine toxins.
  - Estrogen/androgen receptor-mediated toxicities to be addressed first.
- Development of priority assays
  - Identification of test species to act as human surrogates and wildlife sentinels.
  - Agreement on the necessity for *in vivo* assays in this branch of toxicology.
  - Development of *in vitro* and *in vivo* assays, with attention given to their practicality, reproducibility, metabolic capacity, cost, and mechanistic plausibility.
  - Definition of the relationship between *in vitro* assay data and *in vivo* assay data in terms of their value for risk assessment.
- Development of regulations
  - International agreement on a preliminary regulatory strategy, to be developed and further refined with the acquisition of new data.

The divergent testing strategies and regulatory requirements that resulted from individual nations' approaches to carcinogen screening should act as a particular warning. However, the task faced on this occasion is even more complex, because the chemical disturbance of essentially any aspect of animal physiology is under consideration. The detailed knowledge required to respond optimally to this situation is concentrated in a relatively small number of endocrinologists around the world, but they may not be equipped to advance this broad area of toxicology unaided. Thus, there is the need for toxicologists, regulators, and endocrinologists to pool their differing expertise at the international level.

Key among the priorities suggested above are the need to continue to support studies to better define the reality and nature of the hazards posed, and to further investigate the original question of exposure of wildlife and humans to estrogen and androgen receptor agonists/antagonists. When progress has been made in these areas, attention can be given to the development of assays for other mechanisms of endocrine disruption. This will involve the development of assays that measure enzyme or hormone levels and activities *in vivo*. Such techniques may be difficult to refine into robust regulatory tests. This sequential approach to the many issues posed will enable coordinated progress to be made in defined areas. As general confidence in a core set of assays grows, consideration should be given to integrating endpoints into a reduced number of assays.

Finally, one should always keep in mind that the regulation of synthetic chemicals for endocrine disrupting properties may not alleviate the observed increases in human breast and testicular cancer or the apparent decrease in human sperm counts and sperm quality reported for some countries. Therefore, while attending to one possible contributor to these problems—synthetic chemicals—we should remain alert to the possible importance of alternative contributory factors, such as diet and lifestyle.

References

1. Colborn T, Clement C, eds. Chemically-induced alterations in sexual and functional development. Princeton, NJ:Princeton Scientific Publishing, 1992.
2. McLachlan JA. Functional toxicology: a new approach to detect biologically active xenobiotics. Environ Health Perspect 101:386–387 (1993).
3. Colborn T, vom Saal FS, Soto AM. Developmental effect of endocrine disrupting chemicals in wildlife and humans. Environ Health Perspect 101:378–385 (1993).
4. McLachlan JA, Korach KS, eds. Symposium on estrogens in the environment. III: Global health implications. Environ Health Perspect 103(suppl 7):3–178 (1995).
5. National Academy of Sciences. Responsible science: ensuring the integrity of the research process, vol 1. Washington, DC:National Academy Press, 1992.
6. ECETOC. Environmental oestrogens: a compendium of test methods. ECETOC Doc 33. Brussels:European Centre for Ecotoxicology and Toxicology of Chemicals, 1996.
7. Bliley R. Food Quality Protection Act of 1996. 104th Congress, 2nd session. Report 104-669, part 2. Washington, DC:Government Printing Office, 1996;1–89.
8. Kavlock RJ, Daston GP, DeRosa C, Fenner-Crisp P, Gray LE, Kaattari S, Lucier G, Luster M, Mac MJ, Maczka C, et al. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. Environ Health Perspect 104(suppl 4):715–740 (1996).
9. Hill AB. The environment and disease: association or causation? Proc R Soc Med 58:295–300 (1965).
10. Ames BN, Gold LS. Too many rodent carcinogens. Science 249:970–971 (1990).
11. Purdom CE, Hardiman PA, Bye VJ, Eno NC, Tyler CR, Sumpter JP. Estrogenic effects of effluents from sewage treatment works. Chem Ecol 18:275–285 (1994).
12. Harries JE, Sheahan DA, Jobling S, Matthiessen P, Neall P, Routledge EJ, Rycroft R, Sumpter J, Tylor T. A survey of estrogenic activity in inland waters. Environ Toxicol Chem 15:1993–2002 (1996).
13. Abel M, Giger W, Schaffner C. Behavior of alkylphenol polyethoxylate surfactants in the aquatic environment. II. Occurrence and transformation in rivers. Water Res 28:1143–1152 (1994).
14. White R, Jobling S, Hoare SA, Sumpter JP, Parker MG. Environmental persistent alkylphenolic compounds are estrogenic. Endocrinology 135:175–182 (1994).
15. Jobling S, Sheahan D, Osborne JA, Matthiesen P, Sumpter JP. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. Environ Toxicol Chem 15:194–202 (1995).
16. Brighty G. The identification and assessment of oestrogenic substances in sewage treatment works effluents. R & D technical summary P38. Bristol, U.K.:U.K. Environmental Agency, 1996;1–4.
17. Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serano F. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ Health Perspect 103(suppl 7):113–122 (1995).
18. Zakarewski T. A review of *in vitro* bioassays for assessing estrogenic substances. Environ Sci Technol (in press).
19. Sharpe RM, Fisher JS, Millar MM, Jobling S, Sumpter JP. Gestational and lactational exposure of rats to xenoestrogens results in reduced testicular size and sperm production. Environ Health Perspect 103:1136–1143 (1995).
20. Meek MD, Clemons J, Wu ZF, Zackerewski TB, Martins MA. Assessment of the alleged estrogen receptor-mediated activity of phthalate esters [Abstract 443]. Presented at the 17th Annual SETAC Meeting, 24–25 November 1996, Washington, DC.
21. Colerangle JB, Roy D. Exposure of environmental estrogenic compound nonylphenol to noble rats alters cell-cycle kinetics in the mammary gland. Endocrine 4:115–122 (1996).

22. Lee PC, Lee W. *In vivo* estrogenic action of nonyl phenol in immature female rats. Bull Environ Contam Toxicol 57:341–348 (1996).
23. Evans GL, Bryant HU, Magee DE, Turner RT. Raloxifene inhibits bone turnover and prevents further cancellous bone loss in adult ovariectomized rats with established osteopenia. Endocrinology 137:4139–4144 (1996).
24. Yang NN, Venugopalan M, Hardikar S, Glasebrook A. Identification of an estrogen response element activated by metabolites of 17β-estradiol and raloxifene. Science 273: 1222–1225 (1996).
25. Purchase IFH. An international reference chemical data bank would accelerate the development, validation and regulatory acceptance of alternative toxicology tests. ATLA 18:345–348 (1990).
26. ECETOC. Skin irritation and corrosion: reference chemicals data bank. ECETOC Technical Rep No 66. Brussels:European Centre for Ecotoxicology and Toxicology of Chemicals, 1995.
27. Katzenellenbogen JA. The structural pervasiveness of estrogenic activity. Environ Health Perspect 103(suppl 7):99–101 (1995).
28. Waller CL, Oprea TI, Chae K, Park H-K, Korach KS, Laws SC, Wiese TE, Kelce WR, Gray LE Jr. Ligand-based identification of environmental estrogens. Chem Res Toxicol 9:1240–1248 (1996).
29. Ren L, Medahl A, Lech JJ. Dimethyl formamide (DMFA) and ethylene glycol (EG) are estrogenic in rainbow trout. Chem Biol Interact 102:63–67 (1996)
30. Mellanen P, Petanen T, Lehtimaki J, Makela S, Bylund G, Holmbom B, Mannila E, Oikari A, Santti R. Wood-derived estrogens: studies *in vitro* with breast cancer cell lines and *in vivo* in trout. Toxicol Appl Pharmacol 136:381–388 (1996).
31. Jobling S, Sumpter JP. Detergent components in sewage effluent are weakly oestrogenic to fish: an *in vitro* study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Aquat Toxicol 27:361–372 (1993).
32. Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J.A. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93:5925–5930 (1996).
33. Shelby MD, Newbold RR, Tully DB, Chae K, Davis VL. Assessing environmental chemicals for estrogenicity using a combination of *in vitro* and *in vivo* assays. Environ Health Perspect 104:1296–1300 (1996).
34. Ashby J, Lefevre PA, Odum J, Harris CA, Routledge EJ, Sumpter JP. Synergism between synthetic oestrogens? Nature 385:494 (1997).
35. Villalobos M, Olea N, Brotons JA, Olea-Serrano MF, Ruiz de Almodovar JM, Pedraza V. The E-SCREEN assay: a comparison of different MCF7 cell stocks. Environ Health Perspect 103: 844–850 (1995).
36. Ramamoorthy K, Wang F, Chen I-C, Norris JD, McDonnell DP, Leonard LS, Gaido KW, Bocchinfuso WP, Korach KS, Safe S. Estrogenic activity of a dieldrin/toxaphene mixture in the mouse uterus, MCF-7 human breast cancer cells and yeast-based estrogen receptor assays: no apparent synergism. Endocrinology (in press).
37. Arnold SF, Klotz DM, Collins BM, Vonier PM, Guillette Jr. LJ, McLachlan JA. Synergistic activation of estrogen receptor with combinations of environmental chemicals. Science 272:1489–1492 (1996).
38. Reel JR, Lamb JC, Neal BH. Survey and assessment of mammalian estrogen biological assays for hazard characterization. Fundam Appl Toxicol 34:288–305 (1996).
39. O'Connor JC, Cook JC, Craven SC, Van Pelt CS, Obourn JD. An *in vivo* battery for identifying endocrine modulators that are estrogenic or dopamine regulators. Fundam Appl Toxicol 33:182–195 (1996).
40. Teng C. Mouse lactoferrin gene: a marker for estrogen and epidermal growth factor. Environ Health Perspect 103(suppl 7):17–20 (1995).
41. Ashby J. Endocrine modulation of human reproduction by environmental chemicals. Environ Toxicol Pharmacol (in press).
42. Cooke PS, Zhao Y, Hansen LG. Neonatal polychlorinated biphenyl treatment increases adult testis size and sperm production in the rat. Toxicol Appl Pharmacol 136:112–117 (1996).
43. Li M-H, Hansen LG. Responses of prepubertal female rats to environmental PCBs with high and low dioxin equivalencies. Fundam Appl Toxicol 33:282–293 (1996).
44. Kelce WR, Wong C, Gray LE Jr, Wilson EM. Environmental antiandrogens. Fundam Appl Toxicol 2: 9–11 (1996).